Inhibition of Electron Transport Enzymes and Cholinesterases by Endrin

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Biochemical investigations on the action of chlorinated hydrocarbon insecticides have been scarce until recently and even now the majority of such studies deals only with DDT and its derivatives. No clear answers to the site of action or to the primary effect of chlorinated insecticides such as aldrin, endrin and dieldrin have been presented although several investigations have suggested possible effects on enzyme systems. Morrison and Brown (1) studied the activity of cytochrome oxidase from the cockroach in the presence of aldrin and dieldrin, and concluded that inhibition of activity was complete at insecticide concentrations of 1 mM. Sova (2) has demonstrated a small inhibition of lactate dehydrogenase from rabbit muscle by endrin and dieldrin at concentrations of 0.1 to 1 mM when following NAD reduction spectrophotometrically. Stimulation was noted if a tetrazolium colorimetric method was employed. Hayama and Kuwabasa (3) reported that slight inhibition (2 to 8%) of serum

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Bulletin of Environmental Contamination & Toxicology,
Vol. 3, No. 2, 1968, published by Springer-Verlag New York Inc.

cholinesterase occurred at concentrations of 5 to 50 ppb of endrin in blood serum; however, channel catfish killed by the action of endrin appear to have suffered no loss in brain acetylchloinesterase activity due to endrin exposure (4). We wish to present data supporting certain of these reports and extending the observations to include the effects of endrin on other major components of the terminal oxidation system from a highly susceptible organism, the catfish, Ictalurus melas.

Experimental Methods

Fish were collected from local lakes and retained in large laboratory aquaria for two weeks prior to sacrifice. Fresh livers, brain or spinal cord were excised and placed in iced dishes containing 0.3 M Tris-HCl buffer, pH 7.6. Homogenization was achieved by 1 to 2 minutes of grinding in 0.25 M sucrose in a glass tissue grinder fitted with a teflon pestle. Liver mitochondria were prepared by the density gradient method of Hogeboom (5). If disrupted mitochondria were desired, they were obtained by two 30-second treatments with a Branson sonifier. In some instances, samples were stored overnight at 4°C before use.

Succinate dehydrogenase was assayed manometrically by the procedure of Bernath and Singer (6). NADH-cytochrome c reductase was measured spectrophotometrically according to Brodie (7). Cytochrome oxidase was estimated by the manometric method of Schneider and Potter (8). Acetylcholinesterase was assayed specifically by the colorimetric method of Hestrin (9), with acetyl-β-methyl choline as substrate. A less specific procedure,

that described by Ellman, et al. (10) wherein acetylthiocholine is substrate, was also employed.

Protein was measured by the method of Lowry, et al. (11). Actual endrin concentrations were estimated by gas chromatographic analysis of stock solutions used for preparation of diluted samples. Endrin was added to all reaction mixtures either as a saturated solution in the appropriate buffer or dissolved in a 5% acetone, 5% alcohol, 0.5% Triton X-100 aqueous solution. In the former method, the endrin final concentration was approximately 0.5 μM. The latter method permitted employing endrin concentrations up to 80 μM.

All manometric assays were performed in 3.0 ml volume at 37°C with air as the gas phase. All spectrophotometric assays were done at 25°C in 3.0 ml volume in a Beckman DB spectrophotometer equipped with a logarithmic recorder. Control reactions without substrate always contained the organic solvent mixture whenever appropriate. The volume of inhibitor solution added was 0.25 ml in most cases. Increases in volume of inhibitor solution above this level resulted in a marked inconsistency in activity values. Assays were usually conducted in triplicate, wherein variation was slight. Averaging of results from different preparations or from the same preparation stored for varying periods was not attempted, as discussed later.

Results

Assays of acetylcholinesterase in brain-spinal cord homogenates revealed no inhibition by endrin at a final concentration

of 10 μM when acetyl-β-methyl choline was substrate. This substrate is not hydrolyzed significantly by pseudocholinesterases, but only by true acetylcholinesterase (12). Table I illustrates results when acetylthiocholine was employed as substrate. If quinidine sulfate was added to inhibit pseudocholinesterase activity, again no inhibition of acetylcholinesterase by endrin was seen. Omission of quinidine sulfate permitted demonstration of a slight inhibition due to endrin. This suggests that the inhibitory effect is exerted only on the pseudocholinesterase activity, in agreement with the earlier reports on this enzyme in serum (3).

TABLE I

Acetylthiocholine Hydrolysis by Brain-Spinal Cord Homogenate

	Activity (Δ OD/min)		
Homogenate	-Endrin	+Endrin	Percent Inhibition
-Quinidine sulfate	0.125	0.113	10%
+Quinidine sulfate	0.060	0.060	0%

Assays were performed as described in the text. Quinidine sulfate (0.5 mg) was added to the assay when indicated to yield a concentration which totally inhibited the pseudocholinesterase activity. Endrin was added as a saturated aqueous solution, with a final concentration of 0.5 μM. These results are the average of triplicate analyses, with a standard deviation of 0.004 or less.

NADH-cytochrome c reductase from either whole mitochondria or sonic-treated mitochondria which had been sedimented at 144,000 x g and resuspended for assay failed to show any

inhibition at endrin concentrations up to 10 μM.

Limited inhibition was observed with succinate dehydrogenase when measurements were conducted at an endrin concentration of 0.5 μM (no organic solvent present). Inhibition varied from 4 to 10% in 16 separate assays conducted on 5 different mitochondria preparations. Preincubation of mitochondria and endrin at 0°C for times up to 4 hours did not significantly increase the inhibition. Table II summarizes results obtained when higher endrin concentrations were employed. Relatively large inhibitions could be exhibited under these conditions but the inhibition was inversely related to the specific activity of the preparation. All measurements were made at nearly equal enzyme concentrations, however, and the results expressed as specific activity for comparison. Thus accuracy of measurement was

TABLE II

Inhibition of Succinate Dehydrogenase Activity by Endrin Administered in an Organic Solvent Mixture

Preparation	Specific Activity		Endrin (μM)	Percent Inhibition
	-Endrin	+Endrin		
1	19.8 ± 1.30	17.3 ± 1.00	8	12.6
	19.8 ± 1.10	15.0 ± 0.50	80	24.2
2	15.9 ± 0.12	10.9 ± 0.21	8	31.5
	15.9 ± 0.11	6.5 ± 1.41	80	59.1

Specific activity is expressed as μl O_2/5 min/mg protein ± the standard deviation for triplicate analyses.

constant for all assays. It appears conclusive, nonetheless, that endrin is inhibitory; since the highest specific activity obtained was 20 μl O_2/5 min/mg protein, such preparations illustrate minimal inhibition at the chosen endrin concentrations.

Cytochrome oxidase was inhibited when endrin was supplied in aqueous solution or in an organic solvent mixture. In Table III are presented representative data. As with succinate dehydrogenase, the degree of inhibition depended upon both the endrin concentration and the specific activity of the enzyme preparation.

TABLE III

Effect of Endrin on the Specific Activity of Cytochrome Oxidase from Different Mitochondrial Preparations

-Endrin	+Endrin	Endrin Conc. (μM)	Percent Inhibition
12.56 ± 0.05	11.90 ± 0.22	0.5	5
4.49 ± 0.11	3.77 ± 0.02	0.5	16
3.31 ± 0.04	2.67 ± 0.04	0.5	20
13.50 ± 0.7	10.00 ± 0.4	80	27
7.81 ± 0.70	5.06 ± 0.16	80	36
3.30 ± 0.28	1.95 ± 0.05	80	41

Specific activity is expressed as μl O_2/5 min/mg protein ± the standard deviation for triplicate analyses. Endrin was added in aqueous solution (0.5 μM samples) or in an organic solvent mixture (80 μM samples).

The maximum specific activities observed in this system were approximately 13 μl O_2/5 min/mg protein. Even at this activity, a 27% inhibition was noted with endrin present at 80 μM.

Figure 1 illustrates the concentration dependence of the inhibition, with data being collected on a single preparation treated with varying amounts of endrin. Although the quantitative values on the curve were markedly influenced by the activity of the enzyme preparation, a qualitatively similar type of curve was obtained regardless of the preparation used.

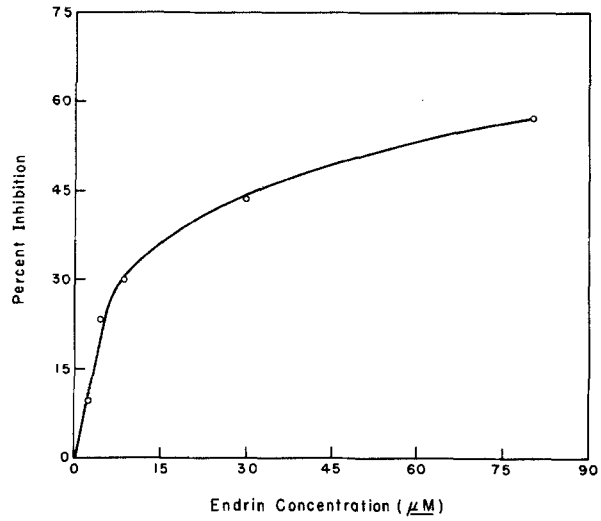

FIG. 1
Concentration Dependence of Endrin Inhibition of Cytochrome Oxidase. All measurements were made on a single mitochondrial preparation having a specific activity of 2.21 μl O_2/5 min/mg protein in the absence of added endrin. Each point represents the average of triplicate determinations. Endrin was added in an organic solvent mixture in each case.

Discussion

Kinetic analysis of the nature of the inhibition exhibited with succinate dehydrogenase and cytochrome oxidase has not been attempted, but it is apparent that endrin does exert some type of detrimental effect on the catalytic activity of these enzymes. The sizeable dependence of inhibition on the specific activity of

the preparation suggests that the structural arrangement of the electron carriers plays a critical role in inhibition by endrin. The high lipid solubility of endrin should facilitate its penetration of the mitochondrial membrane but would tend to promote its retention in lipid layers rather than in the inner protein layer containing the oxidative enzymes. Swelling of mitochondria by storage in aqueous solution results in a decrease in oxidative activity, possibly through alteration of the arrangement of the electron coupling enzymes. Thus storage produces decreased specific activity but may also favor endrin-enzyme interaction through changes in the membrane lipid bilayer by permitting a more ready access to the oxidative enzymes and carriers.

The possibility that endrin exerts an effect on electron transport enzymes by its ability to associate with lipoprotein components of the mitochondrion raises the question of whether the phosphorylative capacity of endrin-treated mitochondria is altered. The uncoupling action of other lipophilic agents such as free fatty acids is well known in mammalian systems (13). Preliminary experiments on phosphorylation in rat liver mitochondria treated with endrin (0.5 μM final concentration) revealed that ATP formation, measured as $^{32}PO_4$ incorporation into glucose-6-phosphate in the presence of excess hexokinase, was inhibited up to 10%, while acetoacetate production from β-hydroxybutyrate was not detectably affected. Further experiments with fish liver mitochondria and higher endrin

concentrations will be necessary before a definite effect on phosphorylative ability can be established.

Although certain evidence has been reported to support nerve components as the principal site of action of such pesticides as dieldrin and endrin (14), it would appear from the present work that any inhibition by these agents to the transmission of nerve impulses would be due to binding of the pesticide to essential components and not directly due either to inhibition or stimulation of acetylcholinesterase activity itself.

We favor the idea that endrin and its analogues bind to lipid-rich structural components of mitochondria and certain other organelles. This binding may subsquently affect the activity of enzymes associated directly with the lipid-rich fractions in those cases where integrity of the structural component is necessary for maximum catalytic activity. The significance of the effects reported here and support for the proposed mode of pesticide lethality can only be realized after study of the affected enzyme systems in submitochondrial preparations and in animals treated *in vivo* with lethal doses of endrin.

Summary

Two enzymes involved in mitochondrial electron transport in the catfish, *Ictalurus melas*, have been shown to be inhibited by endrin. Succinate dehydrogenase and cytochrome oxidase were both inhibited, and the extent of inhibition depended upon endrin concentration and the specific activity of the enzyme preparation.

No appreciable effect of endrin on acetylcholinesterase or NADH-cytochrome c reductase was seen. Binding to lipoprotein components essential for mitochondrial oxidation has been proposed as a logical site for endrin action.

References

1. P. E. Morrison and A. S. A. Brown, J. Econ. Entom. 47, 723 (1954).
2. C. R. Sova, Science 154, 1661 (1966).
3. K. Hayama and S. Kuwabasa, Nippon Suisan Gakkaish 28, 179 (1962).
4. D. I. Mount and G. J. Putnicki, Summary Report of the 1963 Mississippi River Fish Kill Investigation, Wildlife and Nat. Res. Conf., Pittsburgh, Pa., (1966).
5. G. H. Hogeboom, Methods in Enzymology, Vol. 1, S. P. Colowick and N. O. Kaplan, Eds., p. 16, Academic Press, New York (1955).
6. P. Bernath and T. P. Singer, Methods in Enzymology, Vol. 1, S. P. Colowick and N. O. Kaplan, Eds., p. 597, Academic Press, New York (1955).
7. A. F. Brodie, Methods in Enzymology, Vol. 2, S. P. Colowick and N. O. Kaplan, Eds., p. 693, Academic Press, New York (1955).
8. W. R. Schneider and V. R. Potter, J. Biol. Chem. 149, 217 (1943).
9. S. Hestrin, J. Biol. Chem. 180, 249 (1949).
10. G. L. Ellman, K. D. Courtney, V. Andres, and R. M. Featherstone, Biochem. Pharmacol. 7, 88 (1961).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. 193, 265 (1951).
12. D. Nachmansohn and I. B. Wilson, Methods in Enzymology, Vol. 1, S. P. Colowick and N. O. Kaplan, Eds., p. 644, Academic Press, New York (1955).
13. A. L. Lehninger, The Mitochondrion, p. 92, W. A. Benjamin, Inc., New York (1964).
14. F. Matsumura and M. Hayaishi, Science 153, 757 (1966).